

Efficient Production and Processing of Elastase and LasA by *Pseudomonas aeruginosa* Require Zinc and Calcium Ions

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The ability of *Pseudomonas aeruginosa* to degrade elastin, a major component of connective tissue, likely contributes to its pathogenicity and multiplication in human tissues. Two extracellular enzymes are required for *P. aeruginosa* elastolytic activity: elastase and LasA. Elastase is a zinc metalloprotease, but little is known about the structure of LasA. When grown under metal ion-deficient conditions, *P. aeruginosa* culture supernatants were found to exhibit a low level of elastolytic activity, which coincided with production of low levels of the 51-kDa proelastase and no detectable LasA. By using this fact to identify factors that promote elastolytic activity, *P. aeruginosa* PAO1, FRD2, and DG1 were grown in metal ion-deficient medium supplemented with zinc (10^{-4} M ZnCl_2), calcium (2.5×10^{-3} M CaCl_2), or iron (10^{-4} M FeCl_3). High levels of proteolytic and elastolytic activity were exhibited by all strains when cultured in the presence of both zinc and calcium, and this was associated with the production of mature 33-kDa elastase and 21-kDa LasA. Supplementing DG1 and PAO1 cultures with zinc alone stimulated the production of 33-kDa elastase, which, because of the calcium-deficient conditions, exhibited low proteolytic and elastolytic activities. Zinc also stimulated the production of a 41-kDa form of LasA in DG1 and PAO1 culture supernatants. Elastase production by FRD2 cultured in the presence of zinc alone differed from that by the other two strains in that supernatants contained 33-kDa elastase, a 21-kDa form of LasA, and exhibited high proteolytic and elastolytic activities. Such strain-associated differences in LasA processing and elastase activity can be explained by differences in metal ion-scavenging mechanisms adapted by the strains. Supplementing cultures with calcium stimulated the production of elastase but had no effect on LasA production. The elastase produced exhibited variable sizes, possibly resulting from aberrant processing reactions, and showed little proteolytic activity. Proteolytic activity could be recovered from 33-kDa elastase produced in the presence of calcium by inclusion of zinc in the enzymatic assay. Although iron was previously found to exert a repressive effect on *P. aeruginosa* elastolytic activity, iron exerted little effect on elastolytic activity when added to cultures containing both zinc and calcium. These studies support the conclusion that elastase production and processing are promoted by both zinc and calcium. LasA production, in comparison, is stimulated by zinc, with both zinc and calcium facilitating its processing. The association of 41-kDa LasA with a low level of elastolytic activity and of 21-kDa LasA with a high level of activity supports the conclusion that *lasA* encodes a larger, precursor protein which is processed to an active 21-kDa form during secretion.

Pseudomonas aeruginosa is an opportunistic pathogen known to cause severe and lethal infections in compromised hosts. Elastase is one of several extracellular enzymes produced by *P. aeruginosa* that is believed to contribute to its pathogenesis. The role of elastase as a virulence factor is supported by its substrate repertoire, which includes elastin (21), some collagens (12), human immunoglobulins G and A (5, 11), serum α_1 -proteinase inhibitor (23), and complement components (29).

Pseudomonas elastase is a neutral metalloprotease, requiring zinc for enzymatic reactivity and calcium for stability (21). The sequence (1, 6, 31) and crystallographic structure (32) of elastase are known. It shares a high degree of sequence and functional homology with thermolysin, a zinc metalloprotease produced by *Bacillus thermoproteolyticus*. Specific residues involved in elastase enzymatic activity, zinc binding, and calcium binding were predicted on the basis of comparisons with the thermolysin structure. Encoded by the *lasB* gene, elastase is synthesized as a pre-proenzyme of 53 kDa, with translocation through the inner

membrane leading to removal of a signal sequence and formation of 51-kDa proelastase (16). Proelastase is then further processed to its mature 33-kDa form via autoproteolytic processing mechanisms. In this regard, McIver et al. (20) have recently shown that substitutions at the active site residue His-223 resulted in both loss of proteolytic activity and accumulation of the unprocessed 51-kDa form of elastase when expressed in *Escherichia coli* (22).

Although the structure of elastase has been characterized, less is known about the regulation of elastase production. Several environmental factors, including iron, ammonium chloride, glucose (2, 15), and zinc (3), have been implicated in the regulation of elastase production. In addition, a gene required for the transcription of elastase, *lasR*, has been identified elsewhere (7). Elastolytic activity of *P. aeruginosa* elastase is also greatly enhanced by a second protein, LasA (9). Expression of *lasA* in *E. coli* resulted in the production of a protein of approximately 41 kDa (28). However, recent studies show that LasA is secreted as a 21-kDa fragment from *P. aeruginosa* (27). This suggests that, like elastase, LasA may be synthesized as a larger precursor which undergoes proteolytic processing during secretion. Although LasA has no apparent proteolytic activity on elastin, it may

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facilitate the elastolytic activity of elastase by allowing normally inaccessible sites within elastin to be exposed to the elastolytic activity of elastase (27).

In this study, we examined the roles of iron, zinc, and calcium ions in the production of elastase and LasA in *P. aeruginosa* culture supernatants. Our results showed that zinc and calcium promoted efficient production and processing of both active elastase and active LasA.

MATERIALS AND METHODS

Bacterial strains. Prototrophic strains of *P. aeruginosa* used in this study include PAO1 (14), DG1 (34), FRD2, a spontaneous nonmucoid revertant of the cystic fibrosis isolate FRD1 (8), PA103, an elastase-negative strain (25), and PAO-E64, a *lasA1* mutant of PAO1 which is elastolytic deficient (24).

Media. Bacteria were cultured in a nutrient-rich medium, 2YT (1% yeast extract, 1.6% tryptone, 1% NaCl), or a nutrient-restricted medium, TSBD (25). The latter was prepared by treating 30 g of Trypticase soy broth (BBL Microbiology Systems) in 90 ml of water with 10 g of Chelex-100 (minus 400-mesh; Bio-Rad Laboratories). Chelex-100, an ion exchange resin used to remove metal ions, was stirred with the medium for 6 h at room temperature. The chelexed medium was then dialyzed against 1 liter of deionized water at 5°C for 15 h, sterilized, and supplemented with 1% glycerol and 0.05 M monosodium glutamate (Sigma). When indicated, the following metals were added to TSBD before inoculation of bacteria: Zn (10^{-4} M ZnCl_2), Ca (2.5×10^{-3} M CaCl_2), and Fe (10^{-4} M FeCl_3). Metal ion concentrations were chosen on the basis of previous metalloprotease (19) and exotoxin A studies (2).

Culture conditions. All cultures were grown at 37°C for 18 h in a reciprocating water bath at 100 rpm. For studies on the influence of metal ions on elastase and LasA production, cultures containing different ions were grown simultaneously, harvested, and processed under identical conditions. Upon harvest, 1.0 ml of each culture was removed and used to prepare a cell extract fraction by centrifuging at $12,000 \times g$ for 10 min, resuspending the pellet in Laemmli sample buffer (18) containing 5% 2-mercaptoethanol (Bio-Rad), and immediately incubating at 100°C for 5 min. The remainder of the culture was centrifuged at $12,000 \times g$ for 15 min at 4°C. A sample of each culture supernatant was immediately frozen at -70°C to be later assayed for enzyme activity. The remaining supernatant was then concentrated 10-fold by using a Minicon device (Amicon) and processed for polyacrylamide gel electrophoresis (PAGE) according to the method of Laemmli (18). During all processing procedures, supernatants were maintained at 4°C and protected from proteolysis by the addition of 1 mM phenylmethylsulfonyl fluoride (Sigma) and 5 mM EDTA. The addition of EDTA during processing manipulations was found to greatly enhance the integrity of proteins in culture supernatants.

SDS-PAGE and immunoblot analysis. To assess production and processing of elastase and LasA, 10-fold-concentrated supernatant fractions were separated under nonreducing conditions by sodium dodecyl sulfate (SDS)-PAGE on a 4.5% stacking gel and resolved in a 5 to 15% gradient gel (30:0.54 [acrylamide to *N,N*-methylene-bis-acrylamide]). Immunoblots were performed according to the method of Towbin et al. (33). Primary antisera were prepared as follows. Rabbit antielastase was prepared against purified elastase (Nagase Biochemicals), which was diluted in 5 mM Tris-HCl-10 mM EDTA (pH 7.5). Rat anti-LasA was pre-

pared against a synthetic peptide representing a 22-residue, hydrophilic segment of LasA. The peptide was identical to residues 77 to 98 of the inferred amino acid sequence of the secreted fragment of LasA (TNYHMDQIQVSNQGV SADTK) (4) and was synthesized by an automated peptide synthesizer (Beckman Instruments) and a solid-phase method (30). A C-terminal cysteine residue added to the peptide during synthesis permitted peptide cross-linking. To immunize, the cross-linked peptide was absorbed onto nitrocellulose, and the nitrocellulose was transferred to the peritoneal cavity of rats with an 18-gauge needle and stylet (26). This method proved effective in inducing LasA cross-reactive antibodies in the absence of a carrier protein. Six injections of 50 μg of peptide were given during a 1-year immunization period. Cross-reactivity with a single 21-kDa protein in *P. aeruginosa* culture supernatants was detected by immunoblot analysis after the third injection of immunogen. This reactivity increased slightly upon additional immunizations.

For immunoblot analysis, rabbit anti-elastase serum was used at a 1:800 dilution, with a 1:1,000 dilution of peroxidase-conjugated goat anti-rabbit (Sigma) as the secondary antibody. Rat anti-LasA was used at a 1:200 dilution, with a 1:1,000 dilution of goat anti-rat (Sigma) as the secondary antibody. All immunoblot results were confirmed in repeated culturing studies and in repeated immunoblot analyses.

Protease and elastase enzyme assays. Levels of proteolytic activity of unconcentrated culture supernatants were determined by an azocasein assay previously described (17). Briefly, 50 μl of culture supernatant was added to 1.0 ml of 0.3% azocasein (Sigma) in a solution containing 5 mM Tris-HCl and 0.5 mM CaCl_2 (pH 7.5). The reaction mixture was incubated for 30 min at 37°C. Undigested substrate was precipitated with 3.3% trichloroacetic acid and removed by centrifugation ($10,000 \times g$ for 10 min). The A_{400} of the supernatant was measured, with units of proteolytic activity expressed as the increase in A_{400} per milligram of total protein. Elastolytic activity was performed, as previously described (24), in 3.0 ml of 0.1 M Tris-maleate-1 mM CaCl_2 (pH 7.0) containing 10 mg of elastin Congo red (Sigma). For this assay, 500 μl of unconcentrated culture supernatant was added to the reaction mixture and incubated for 2 h at 37°C with rotation. The reaction was stopped by the addition of 2.0 ml of 0.7 M phosphate buffer (pH 6.0), and undigested elastin was removed by centrifugation ($10,000 \times g$ for 10 min). The A_{495} of the supernatant was measured, with elastolytic units expressed as the increase in A_{495} per milligram of protein. The total protein content of culture supernatants was determined by the bicinchoninic acid protein assay kit purchased from Pierce Biochemical.

Quantitation of elastase and LasA by ELISA. Relative concentrations of elastase and LasA in culture supernatants were quantified in a direct-binding enzyme-linked immunosorbent assay (ELISA), with a modification of methods previously described (26). Since immunoglobulins serve as an elastase substrate, to quantitate proteins in supernatants that contain elastase by ELISA first requires that the protease activity of elastase be inhibited. This inhibition, however, must occur without the loss of immunological integrity of elastase and LasA. To achieve this, culture supernatant aliquots maintained at -70°C were diluted 1:50 in ELISA coating buffer, and 100 μl of each diluted supernatant was added to ELISA wells. The ELISA plate was placed in a 100°C water bath for 3 min and then placed at 4°C overnight. Elastase was quantified by adding 100 μl of a 1:250 dilution of rabbit anti-elastase serum to each well, and LasA was

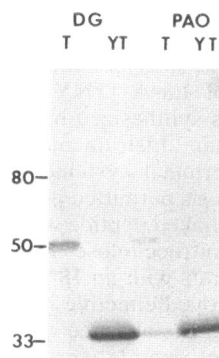


FIG. 1. Immunoblot analysis of elastase production by strains DG1 (DG) and PAO1 (PAO) cultured in either TSBD (T) or 2YT (YT) medium. Proteins in 10-fold-concentrated culture supernatants were separated by PAGE on a 4.5% stacking gel and 5 to 15% gradient gel. The proteins were then immunoblotted, and elastase was detected with an antiserum produced against purified elastase, diluted 1:800. Proelastase is 51 kDa, while mature elastase is 33 kDa.

quantified by adding a 1:100 dilution of rat anti-LasA peptide serum to each well. Addition of a 1:1,000 dilution of peroxidase-conjugated goat anti-rabbit (Cappel) or goat anti-rat (Sigma) followed by the substrate 4-chloro-1-naphthol (Sigma) allowed detection. Under the conditions described above, elastase immunoglobulin protease activity was inhibited, yet both elastase and LasA maintained the potential to be specifically recognized by antibodies as determined by reproducible quantitation of various dilutions of supernatant aliquots. Similar attempts that used EDTA to inhibit elastase proteolytic activity while maintaining antibody reactivity proved unsuccessful. All assays were performed in triplicate, with the mean ELISA optical density at 450 nm (OD_{450}) per milligram of protein of culture supernatants reported.

RESULTS

The effects of metal ions in the growth medium on elastase production and processing. When grown in a typical, rich-culture medium like 2YT, *P. aeruginosa* PAO1, DG1, FRD2, and PAO-E64 produced mature 33-kDa elastase in culture supernatants, as detected by immunoblot analysis. In comparison, when these strains were grown in TSBD (a medium deficient in metal ions), little or no mature 33-kDa elastase was produced. Instead, lower levels of 51-kDa proelastase were detected in culture supernatants. Among the strains tested, the most notable producers of 51-kDa elastase were strains DG1 and FRD2. An immunoblot of elastase-related proteins in culture supernatants of strains DG1 and PAO1 grown in TSBD or 2YT medium is shown in Fig. 1. A nonelastolytic strain, PA103, showed no elastase production by immunoblot analysis when cultured in either 2YT or TSBD medium (data not shown). These initial studies indicated that TSBD medium lacked factors required for efficient production and processing of elastase by normal elastase-producing *P. aeruginosa* strains and therefore could be used as a means of identifying factors required for elastase production.

Since elastase is a zinc metalloprotease and since metal ions had been removed from TSBD medium by chelation, likely factors required for elastase production were metal ions. Zinc (Zn) and calcium (Ca) ions have previously been shown to be required for enzyme activity and stability of elastase, respectively (21, 22). Iron (Fe) (2) and, more

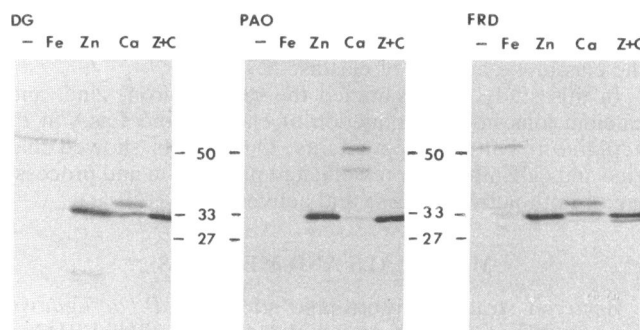


FIG. 2. Immunoblot analysis of elastase production by strains DG2 (DG), PAO1 (PAO), and FRD2 (FRD) grown in TSBD medium with the indicated additions. —, no additions; Fe, 10^{-4} M $FeCl_3$; Zn, 10^{-4} M $ZnCl_2$; Ca, 2.5×10^{-3} M $CaCl_2$; Z+C, 10^{-4} M $ZnCl_2$ plus 2.5×10^{-3} M $CaCl_2$. Proteins from 10-fold-concentrated supernatants were separated by PAGE and immunoblotted as described in the legend to Fig. 1.

recently, Zn (3) have also been implicated in the regulation of elastase production. To determine whether Zn, Ca, or Fe could affect elastase production in TSBD medium, each ion was added to individual TSBD culture flasks prior to inoculation of bacteria, and after 18 h of growth, culture supernatants were concentrated, processed, and assayed for elastase content by immunoblot analysis.

Figure 2 shows the effects of the addition of Fe, Zn, Ca, or Zn plus Ca to TSBD on elastase production by three *P. aeruginosa* strains, DG1, PAO1, and FRD2. On the basis of immunoblot analyses, the addition of Fe to TSBD medium had little or no effect on either the production or processing of elastase, compared with unsupplemented TSBD. When cultured in the presence of Fe alone, low levels of predominantly 51-kDa elastase were detected in culture supernatants of strains DG1 and FRD2, with elastase difficult to detect in PAO1 supernatants. In contrast, the addition of Zn to TSBD led to enhanced production and processing of elastase to its mature 33-kDa form by all three strains. Somewhat similarly, the addition of Ca to TSBD led to enhanced elastase production by all strains; however, proteolytic processing of proelastase in the presence of Ca was incomplete and varied between the strains. Elastase species of 33 and 35 kDa were detected in Ca-TSBD supernatants of strains DG1 and FRD2. In comparison, the majority of the elastase in Ca-TSBD supernatants of PAO1 remained in the 51-kDa proelastase form, with minor 45- and 33-kDa forms detectable. As observed upon the addition of Zn alone to TSBD medium, the addition of both Zn and Ca to TSBD resulted in the production of high levels of mature 33-kDa elastase in culture supernatants by all three strains. These data indicate that Zn stimulated elastase production and led to efficient processing of proelastase to the mature form. Ca also stimulated elastase production, but the addition of Ca alone resulted in elastase cleavage products of variable sizes.

Metal ion effects on elastase production observed by immunoblot analyses were quantitatively confirmed by ELISA. As shown in Table 1, addition of Fe alone to TSBD had little effect on elastase production by any of the three strains. In comparison, the addition of both Zn and Ca increased elastase production to levels which approached or were greater than those observed in 2YT medium. Quantitative results, in combination with immunoblot analyses,

TABLE 1. Quantitation of elastase and LasA in culture supernatants of *P. aeruginosa* strains grown in various culture media

Strain	Culture medium ^a	ELISA OD ₄₅₀ /mg of protein for:	
		Elastase ^b	LasA ^c
DG1	YT	0.075	0.065
	TSBD	0.005	0.040
	TSBD + Fe	0.007	0.065
	TSBD + Zn + Ca	0.186	0.226
	TSBD + Zn + Ca + Fe	0.339	0.529
PAO1	YT	0.252	0.199
	TSBD	0.004	0.021
	TSBD + Fe	0.005	0.023
	TSBD + Zn + Ca	0.168	0.111
	TSBD + Zn + Ca + Fe	0.247	0.176
FRD2	YT	0.166	0.075
	TSBD	0.011	0.054
	TSBD + Fe	0.017	0.081
	TSBD + Zn + Ca	0.241	0.205
	TSBD + Zn + Ca + Fe	0.352	0.354

^a Cultures were grown in 2YT medium or the metal-deficient medium TSBD supplemented with the indicated ions.

^b Elastase concentrations were quantified by ELISA, with the means of assays performed in triplicate represented as ELISA OD₄₅₀ per milligram of protein.

^c LasA concentrations were quantified by ELISA, with the means of assays performed in triplicate represented as ELISA OD₄₅₀ per milligram of protein.

thus support the conclusion that the addition of Zn and Ca to TSBD almost completely restored its potential to support normal elastase production by these *P. aeruginosa* strains.

Although the primary emphasis of these studies was on analysis of secreted elastase, cell-associated forms of elastase in the cultures described above (i.e., both cytoplasmic and periplasmic fractions combined) were also examined for elastase content. On the basis of immunoblot analysis, banding patterns and concentrations of elastase products in cell extracts of cultures grown in TSBD, Fe-TSBD, Zn-TSBD, and Zn-Ca-TSBD closely resembled those observed in the respective culture supernatants (data not shown). In these instances, elastase content in culture supernatants thus closely reflected total elastase production and processing by the organism. The incomplete and variable processing of elastase observed in Ca-TSBD supernatants (Fig. 2) was not, however, as apparent in cell-associated fractions. In the case of DG1 and FRD2, only the 33-kDa form of elastase was observed in cell extracts, not the 35-kDa form also detected in the supernatant. In PAO1 cell extracts, only the 51-kDa proelastase form of elastase was detected, not the 45- or 33-kDa form seen in the supernatant fraction. This suggests that alternative forms of elastase seen in supernatants of Ca-TSBD cultures resulted from extracellular reactions.

The effects of metal ions in the growth medium on LasA production and processing. The elastolytic potential of *P. aeruginosa* culture supernatants is dependent on the production of both LasA and elastase. To detect LasA, a LasA-specific antiserum was prepared using a synthetic peptide defining residues 77 to 98 of the secreted fragment of LasA. This antiserum reacted only with a 21-kDa protein in DG1 and PAO1 culture supernatants grown in 2YT medium. Also as a confirmation of its specificity, the LasA peptide antiserum lacked reactivity with culture supernatant produced by the *lasA1* mutant strain, PAO-E64 (data not shown). This

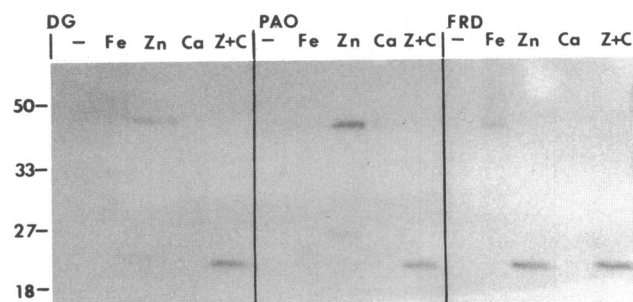


FIG. 3. Immunoblot analysis of LasA production by strains DG1 (DG), PAO1 (PAO), and FRD2 (FRD) grown in TSBD medium in the presence of Fe, Zn, Ca, or Zn plus Ca (Z+C). Additions are as described in the legend to Fig. 2. Proteins from 10-fold-concentrated supernatants were separated by PAGE and immunoblotted as described in the legend to Fig. 1, with a LasA peptide antiserum diluted 1:200. Unprocessed LasA is shown as an ~41-kDa protein, whereas a functional LasA fragment is produced as a 21-kDa protein.

indicates that the elastolytic-deficient phenotype of PAO-E64 may relate to a block in LasA production.

LasA production by strains DG1, PAO1, and FRD2 grown in TSBD in the presence of Fe, Zn, Ca, or Zn plus Ca was evaluated by immunoblot analysis and is shown in Fig. 3. By this method of analysis, no LasA production could be detected in culture supernatants of any of these strains grown in unsupplemented TSBD medium, suggesting that metal ions were required for production of LasA. The addition of Zn alone to TSBD cultures resulted in production of a 41-kDa form of LasA in DG1 and PAO1 supernatants and 21-kDa LasA in FRD2 supernatants. Evidence that the 41-kDa form represents a precursor form of LasA rather than a dimer of the 21-kDa product includes the potential of the cloned *lasA* gene to express a 41-kDa product in *E. coli* (28) and the detection of this 41-kDa product under both reducing and nonreducing SDS-PAGE conditions. The addition of Fe to TSBD resulted in the production of very low levels of an ~41-kDa form of LasA by strains DG1 and FRD2, with no LasA detected in PAO1 supernatants. The addition of Ca alone was not sufficient to produce detectable amounts of LasA by any of the strains. In the presence of both Zn and Ca, all three strains produced the 21-kDa form of LasA. These results suggest that Zn most effectively enhanced LasA production and that both Zn and Ca facilitated processing of LasA to a 21-kDa form.

Quantitation of LasA by ELISA confirmed results observed in immunoblots. As shown in Table 1, the addition of Fe to TSBD resulted in a relative increase in LasA production by strains DG1 and FRD2, with little increase observed by strain PAO1 in TSBD plus Fe (Table 1). Also consistent with immunoblot studies, cultures grown in TSBD plus Zn plus Ca contained approximately four- to fivefold more LasA than cultures grown in TSBD alone. Like elastase, the addition of Zn and Ca to TSBD almost completely restored the potential of this medium to support levels of LasA production comparable to those observed with 2YT medium.

Influence of Zn and Ca in the growth medium on protease activities. Elastase can exhibit both a general protease activity and a more specific elastolytic activity. Supernatants from the cultures described above were examined for both these activities by using two substrates. Azocasein was used to quantitate proteolytic activity, and elastin Congo red was used to quantitate elastolytic activity. For these analyses,

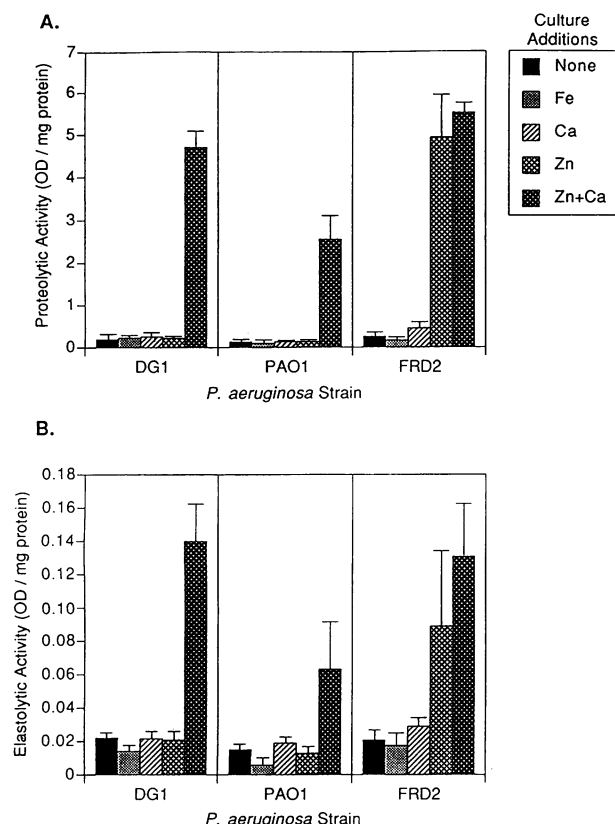


FIG. 4. Protease and elastase enzyme activities associated with culture supernatants of strains DG1, PAO1, and FRD2 grown in TSBD medium with the indicated additions. Notations for additions were as described in the legend to Fig. 2. Protease activity was assayed with an azocasein substrate, and elastase activity was assayed with an elastin Congo red substrate. The means \pm standard deviations of independent culturing studies and independent assays are indicated.

CaCl₂ was added to all assay mixtures to optimize detection of existing enzyme activity (21).

Although the addition of Ca to TSBD resulted in increased production and partial processing of elastase by all strains (Fig. 2), low levels of proteolytic and elastolytic activity were exhibited by culture supernatants (Fig. 4). Similarly, although high levels of mature 33-kDa elastase protein were detected in supernatants of Zn-TSBD cultures (Fig. 2), low proteolytic and elastolytic activities were associated with DG1 and PAO1 supernatants (Fig. 4). These studies showed that the efficient processing of proelastase to its mature 33-kDa form did not necessarily correlate with an increase in enzymatically active elastase. One strain, FRD2, was able to produce high levels of proteolytic and elastolytic activity when cultured in the presence of Zn alone (Fig. 4). This draws attention to strain-related differences in elastase-processing efficiencies, which might relate to differences in their ability to utilize limited metal ion concentrations to produce active elastase. High levels of proteolytic and elastolytic activity were exhibited by all three *P. aeruginosa* strains when grown in TSBD supplemented with both Zn and Ca. Whenever high levels of proteolytic and elastolytic activity were observed, production of 33-kDa elastase and 21-kDa LasA was also observed, indicating that these represented mature, active forms of elastase and LasA.

TABLE 2. Effect of adding zinc to elastolytic and proteolytic assays of *P. aeruginosa* culture supernatants

Strain	Culture addition ^a	Proteolytic activity ^b		Elastolytic activity ^c	
		-Zn	+Zn	-Zn	+Zn
DG1	None	0.180	0.160	0.014	0.017
	Fe	0.200	0.184	0.014	0.012
	Ca	0.239	0.848	0.017	0.017
	Zn + Ca	5.130	4.930	0.167	0.167
PAO1	None	0.120	0.109	0.015	0.012
	Fe	0.088	0.106	0.006	0.015
	Ca	0.131	0.150	0.019	0.013
	Zn + Ca	3.120	2.780	0.090	0.090
FRD2	None	0.250	0.275	0.022	0.020
	Fe	0.160	0.187	0.017	0.032
	Ca	0.438	1.392	0.029	0.029
	Zn + Ca	5.820	5.530	0.166	0.166

^a Cultures were grown in the metal-deficient medium TSBD supplemented with the indicated ions.

^b Proteolytic activity values are increases in OD₄₀₀ per milligram of protein with an azocasein substrate.

^c Elastolytic activity values are increases in OD₄₉₀ per milligram of protein with an elastin Congo red substrate.

Effect of exogenous Zn on enzymatic activity of elastase produced under metal-deficient conditions. To assess the potential of exogenously added Zn to enhance the proteolytic or elastolytic activity of elastase produced in its absence, ZnCl₂ (10⁻⁴ M) was added to the proteolytic and elastolytic assay mixtures prior to assay of supernatants for enzyme activity. The addition of Zn to supernatants of TSBD or Fe-TSBD cultures, which contained 51-kDa proelastase (Fig. 2), showed little if any enhancement of proteolytic activity (Table 2). The addition of Zn to Ca-TSBD culture supernatants of DG1 and FRD2 (which contained mature 33-kDa elastase) resulted in increased proteolytic activity (3.5- and 3.2-fold increases, respectively). Only a minimal increase in protease activity was observed when exogenous Zn was added to Ca-TSBD supernatants of PAO1, consistent with the low levels of 33-kDa elastase. These results suggest that the ability of exogenous Zn to enhance proteolytic activity of cultures deprived of Zn was dependent on the production of 33-kDa elastase. In contrast to its effect on proteolytic activity, exogenous Zn had little effect on elastolytic activity of Zn-deprived culture supernatants (Table 2). This is consistent with the dependency of elastolytic activity on LasA production and the fact that Zn is required during culture for LasA production.

It should be noted that although exogenous Zn was able to enhance proteolytic activity of 33-kDa elastase produced in Ca-TSBD medium, in no instance did exogenous Zn restore protease activity of culture supernatants to levels observed when cultures were grown in the presence of both Zn and Ca. In addition, if exogenous Zn was added to Zn-TSBD or Zn-Ca-TSBD culture supernatants during enzyme assays, no further increases in proteolytic and elastolytic activities were observed. Thus, on the basis of the three metal ions examined in these studies, maximal proteolytic and elastolytic activities were observed when *P. aeruginosa* strains were cultured in the presence of both Zn and Ca.

Influence of Fe on elastolytic activity. Previous studies suggested that the addition of Fe to the culture medium had an inhibitory effect on elastase production by *P. aeruginosa*,

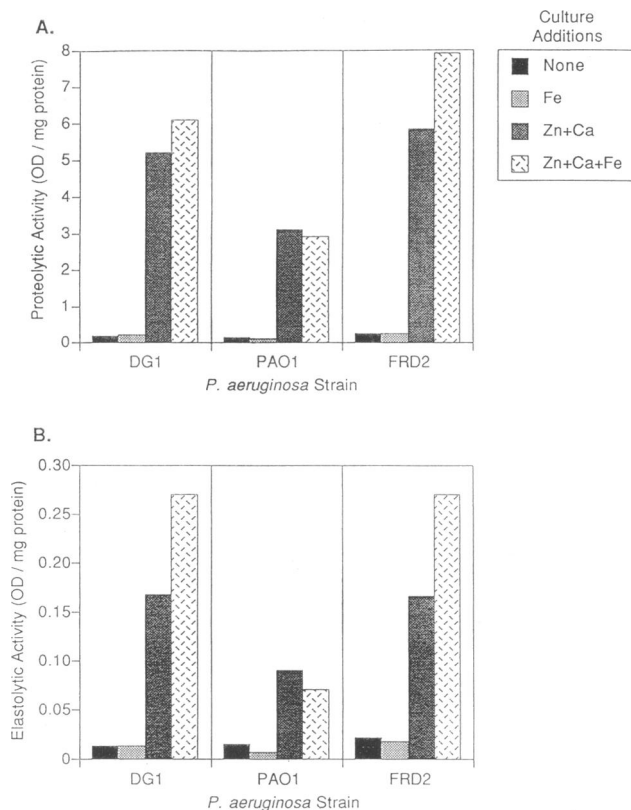


FIG. 5. Protease and elastase enzyme activities associated with culture supernatants of strains DG1, PAO1, and FRD2 grown in TSBD medium in the presence or absence of Fe. Concentrations of Zn, Ca, and Fe added were as described in the legend to Fig. 2. Results were obtained from cultures grown simultaneously in the presence or absence of the indicated ions under identical conditions and harvested and assayed in an identical manner. The results are from several such assays.

similar to the repression exerted by Fe on exotoxin A production (2). TSBD is an Fe-depleted medium originally derived to enhance *P. aeruginosa* exotoxin A production (25). The chelation process used to remove Fe during preparation of TSBD, however, also removes other metal ions, such as Zn and Ca, from the medium. When the elastolytic activities of cultures grown in TSBD with Fe were assayed, low levels of activity, comparable to or slightly lower than those produced in TSBD medium alone, were detected (Fig. 4B). To determine whether Fe had a repressive effect on proteolytic and elastolytic activities when cultures were grown under conditions favoring elastase and LasA production, Fe was added with Zn and Ca to TSBD prior to inoculation of bacteria. As shown in Fig. 5, Fe had no major inhibitory effect on either proteolytic or elastolytic activity when added to Zn-Ca-TSBD growth medium and actually produced what appeared to be an enhancement of enzymatic activity in DG1 and FRD2 culture supernatants. Immunoblot analyses showed no noticeable differences in elastase or LasA production or processing in culture supernatants produced in Zn-Ca-TSBD or Fe-Zn-Ca-TSBD (data not shown). As shown in Table 1, quantitation of elastase and LasA by ELISA also confirmed the lack of an inhibitory effect of Fe on elastase and LasA production when added to Zn-Ca-TSBD medium. In addition, somewhat similarly to

enzyme activity assays, an enhancement of 1.5- to 2.3-fold of the production of both proteins was observed in culture supernatants of all three strains when Fe was added to Zn-Ca-TSBD medium. Thus, although slight decreases in elastolytic activity (most notably with the PAO1 strain) could be detected when Fe alone was added to TSBD medium, in no instance was the inhibitory effect of Fe sufficient to overcome the positive influences exerted by Zn and Ca on elastase enzymatic activity or elastase production. Confirmation of a possible enhancing influence of Fe on elastase or LasA production, when added in combination with Zn and Ca, requires further study.

DISCUSSION

Much of the virulence of *P. aeruginosa* has been attributed to its ability to secrete toxic or degradative enzymes such as elastase. Named after its ability to degrade elastin, elastase is responsible for the majority of the proteolytic activity found in the supernatants of most strains of *P. aeruginosa* (35). Similarly to other metalloproteases, elastase requires zinc for its catalytic reaction (22, 32), is stabilized by calcium ions (21, 32), and appears to be processed via an autoproteolytic mechanism (20). Iron (2) and zinc (3) have been implicated in the regulation of elastase production. Interpretations of studies of the production of elastolytic activity, however, are complicated by the LasA protein, which greatly enhances the elastolytic potential of elastase (9, 24). LasA is secreted into the culture medium, in which it is believed to facilitate the elastolytic activity of elastase by making the elastin substrate more susceptible to proteolysis (27). Mutants with *lasA* defects that lack elastolytic activity retain general proteolytic activity, indicating that LasA may enhance only the elastin-specific enzymatic activity of elastase (9). The *lasA* gene has been shown to be transcriptionally active late in the growth phase (10), but little remains known about environmental factors which promote LasA synthesis and processing.

The studies described here examined the roles of zinc, calcium, and iron in the production of active elastase and LasA. A medium restricted in metal ions (TSBD medium) was found to impair elastase and LasA production and processing. This medium, in turn, was used to identify factors required for efficient production of these two proteins. Three *P. aeruginosa* strains were examined, PAO1, FRD2, and DG1. Testing several strains proved to be valuable in identifying overall trends and strain-dependent effects.

To examine the roles of Zn, Ca, and Fe in elastase and LasA production and processing, each ion was added alone or in combination to TSBD prior to inoculation of 18-h batch cultures. Elastase and LasA proteins were examined in culture supernatants by immunoblot analysis to determine size and relative abundance, with the relative concentrations of both proteins being quantitated by ELISA. The processing of proelastase (51 kDa) to mature elastase (33 kDa) is believed to be autoproteolytic (20). Since Zn and Ca are required for catalysis and stabilization of elastase, reducing the availability of these ions should restrict autoprocessing of proelastase. Consistent with this, low concentrations of unprocessed proelastase were observed in culture supernatants of *P. aeruginosa* strains grown in the metal-deficient TSBD medium. The relative decrease in production of elastase-reactive products in this medium appeared unrelated to possible effects that metal ion deprivation might have on the secretory apparatus, as evidenced by the lack of

intracellular accumulation of elastase and by the efficient secretion of other extracellular proteins, such as exotoxin A (25).

Zinc was shown to play a major role in the elastolytic potential of *P. aeruginosa*. The presence of Zn alone in the culture medium stimulated the production of elastase, which was processed to a mature 33-kDa form in culture supernatants by all strains. Efficient processing and secretion of 33-kDa elastase, however, was not directly associated with high enzymatic activity, as evidenced by DG1 and PAO1 supernatants, which exhibited only low proteolytic activity. This low proteolytic activity was probably related to the inability of these strains to produce stable enzyme when cultured in a Ca-deficient medium. Strain FRD2, in comparison, was able to produce highly enzymatically active elastase in Zn-TSBD medium. This would indicate that strain FRD2 maintained a more efficient means of producing active enzyme under these metal ion-deficient conditions.

LasA production was also stimulated by the addition of Zn to TSBD medium. LasA remained in a 41-kDa form in DG1 and PAO1 supernatants cultured with Zn but was processed to a 21-kDa form by strain FRD2. In previous studies, expression of the *lasA* gene in *E. coli* resulted in production of a 41-kDa protein (28), yet only a 21-kDa form of LasA has previously been identified in *P. aeruginosa* culture supernatants (27). In our studies, both the 21- and 41-kDa forms of LasA were observed in *P. aeruginosa* culture supernatants, with high elastolytic activity associated only with the 21-kDa form. Together, these data provide evidence that *lasA* encodes a larger precursor form of LasA which is proteolytically processed to a mature and active 21-kDa form during secretion from *P. aeruginosa*. The ability of strain FRD2 to process LasA to a 21-kDa active form in the presence of Zn but under Ca-deficient conditions again points to the increased efficiency of this strain in producing active protein under restricted metal ion conditions.

Ca was also found to play a role in proteolytic and elastolytic activity of elastase. Although Ca alone was never shown to stimulate LasA production, supplementing TSBD cultures with Ca led to increased production of elastase. It is unknown at this time whether Ca induced this increase by increasing the rate of transcription from the elastase (*lasB*) promoter or whether another mechanism was involved. Elastase produced in the presence of Ca alone was observed to undergo variable, strain-dependent processing reactions. Culturing in Ca-TSBD medium led to the production of 35- and 33-kDa forms of elastase in DG1 and FRD2 supernatants, while elastase remained primarily in its unprocessed, 51-kDa proelastase form in PAO1 supernatants. These strain variations in elastase processing indicate that under conditions in which other metal ions, such as Zn, are limiting, Ca directs alternative processing mechanisms.

The detection of mature-size but inactive elastase in the supernatants of cultures supplemented with only Ca also prompted studies to determine whether the addition of Zn postsynthesis might reclaim the enzymatic activity of the mature elastase. Consistent with the requirement of Zn for elastase activity, the addition of Zn during the enzyme assay resulted in an approximately threefold stimulation in the proteolytic activity of DG1 and FRD2 supernatants produced in Ca-TSBD cultures. The increase in proteolytic activity occurred, however, without a concomitant increase in elastolytic activity. This lack of elastolytic activity most likely relates to the inefficiency of Ca alone in inducing LasA production.

In all three strains, high levels of proteolytic and elas-

tolytic activities, comparable to those produced in nutrient-rich 2YT medium, were observed when both Zn and Ca were added to TSBD during culture. These high levels of activity also coincided with the production of 33-kDa elastase and 21-kDa LasA. These results support the conclusion of the importance of both Zn and Ca to the production of mature, active elastase and LasA. Our studies, however, also draw attention to strain-related exceptions to this requirement. One possible explanation for these strain variations might relate to differences in the primary amino acid sequence of elastase. Such variations could alter the metal ion requirements of elastase for stabilization and enzymatic activities. Although the complete amino acid sequences of FRD2 and DG1 elastase are not available for direct comparison to that of PAO1, a recent comparison of other elastase sequences suggests that little variation occurs between different strains (31). An alternative explanation for elastase-processing variation could relate to strain differences in metal ion-scavenging mechanisms. For example, the inefficient processing of proelastase by strain PAO1 in Ca-TSBD could result from this strain being less effective than strains DG1 and FRD2 in scavenging the limited Zn ions available. In comparison, the ability of FRD2 to exhibit the highest levels of proteolytic and elastolytic activity in Zn-TSBD could relate to this strain having adapted more efficient mechanisms for scavenging limited Ca ions from this medium. The increased ability of FRD2 to sequester metal ions could, in part, be related to a defect in lipopolysaccharide (LPS) biosynthesis present in this strain. It has previously been observed that isolates from patients with cystic fibrosis (as in strain FRD) often produce rough LPS (13). Since divalent cations are known to play an important role in the structure of LPS, the lack of O side chains in FRD2 might affect its surface-charge density in a manner that would favorably influence the availability of limited metal ions. Since elastase most likely plays a nutritional role in *P. aeruginosa*, a mechanism that permits strains such as FRD2 to have enhanced proteolytic activity in a nutrient-stressed environment should favor their growth under conditions in which metal ions are likely to be limited, as might occur in chronic lung infections.

Iron, in comparison to Zn and Ca, proved to have the least effect on elastase production and processing. Compared with the low elastolytic activity produced in unsupplemented TSBD medium, the addition of Fe to TSBD resulted in comparable or slightly lower levels of activity, similar to those previously reported in studies that used the CaCl_2 precipitation method to deferrate TSBD medium (2). However, when Fe was added to TSBD which also contained Zn and Ca, little effect on elastolytic activity was observed in the resulting culture supernatants. In fact, DG1 and FRD2 culture supernatants showed an enhancement in elastolytic activity when Fe was added to Zn-Ca-TSBD medium. When quantified by an ELISA, an increase in elastase and LasA production was detected in all three strains when Fe was added with Zn and Ca to TSBD. Thus, unlike its inhibitory effect on exotoxin A production, Fe apparently has no major inhibitory role in the elastolytic potential of *P. aeruginosa*.

The studies presented here suggest that the relationship between elastase and LasA extends beyond their functional collaboration in elastolytic activity. Efficient production and processing of both elastase and LasA occurred when *P. aeruginosa* strains were cultured in the presence of both Zn and Ca. Both proteins were produced in a precursor form, with the processing of elastase to a mature 33-kDa form and LasA to a mature 21-kDa form required for high proteolytic and elastolytic activities. While elastase production could be

induced by both Zn and Ca, LasA was induced by only Zn. The dependence on Zn for LasA production would be consistent with Zn being required for LasA activity or being a component of the mature LasA protein.

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